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Acriflavine Inhibits Acquired Drug Resistance by Blocking the Epithelial-to-Mesenchymal Transition and the Unfolded Protein Response

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Abstract

Epithelial-to-mesenchymal transition (EMT) is linked to tumor invasion, drug resistance and aggressive disease and this is largely dependent on the cell's microenvironment. Acriflavine (ACF) is an old antibacterial drug recently also suggested as anticancer agent and HIF inhibitor. We wanted to study the effect of acriflavine on EMT in different human cancer models. Pancreatic cancer cells (Panc-1) were exposed to TGF-β1 or cobalt chloride (to mimick severe hypoxia) to induce EMT. For our third model we exposed HepG2 liver cancer cells to sorafenib which resulted in development of acquired drug resistance with strong features of EMT and aggressive behavior. These models were morphologically and functionally (invasion assay) characterized. Markers of EMT were determined using qRT-PCR and Western blotting. Transcriptome analysis was performed following gene expression determination and combining the iRegulon tool and Gene Set Enrichment Analysis (GSEA). We made the following observations: (1) acriflavine inhibited EMT based on changes in cell morphology, invasive capacities and markers of EMT (at protein and gene expression level). (2) Transcriptome analysis revealed potent inhibition of ATF4 target genes and of the unfolded protein response. We showed that acriflavine blocked eIF2a phosphorylation and reduced ATF4 translation thereby inhibiting the PERK/eIF2a/ATF4 UPR pathway. (3) ACF restored drug sensitivity of cells that obtained acquired resistance. Conclusions: We identified acriflavine as a potent inhibitor of EMT and the UPR, thereby re-sensitizing the cancer cells to antineoplastic drugs.

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Introduction

The effectiveness of systemic cancer therapy is limited by the inevitable emergence of drug resistance. A diverse range of molecular mechanisms are implicated in the reduced treatment responses [1]. EMT is a process in which an epithelial cell, characterized by apical-basal polarity and interaction with a basement membrane, undergoes a shift towards a more motile, invasive state. For a long time, EMT was considered the driving mechanism of the epithelial cancer cell to spread to distant organs and formation of metastatic colonies. This paradigm was very recently challenged by the finding that cells can metastasize also without undergoing EMT [2,3]. In the same studies the important role of the EMT program in drug resistance was confirmed.

The (partial) activation of either the epithelial or the mesenchymal program is largely dependent on the contextual signals that reach the cell from its microenvironment (inflammation, hypoxia, metabolic

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stress etc.). TGF-β1, an inflammatory cytokine mainly produced by macrophages and myofibroblasts, is the most studied inducer of the mesenchymal phenotype [4–6]. Hypoxia has also been reported to induce EMT in cancer cells [7,8]. Furthermore, numerous studies have shown that cell lines derived from various epithelial cancers undergo mesenchymal differentiation in vitro when made resistant to conventional chemotherapy [9–11] as well as to targeted agents [12–14].

In response to stress such as nutrient deprivation, severe hypoxia or pH changes, the tumor cell will also engage additional signaling pathways such as the unfolded protein response (UPR) [15,16]). The UPR is activated when the stressor disrupts normal protein folding in the endoplasmic reticulum (ER) with the aim to ensure survival of the cell. However, when cell stress is too severe and prolonged, the UPR will facilitate apoptosis [15]. One of the main UPR signaling pathways is driven by protein kinase RNA-like endoplasmic reticulum kinase (PERK) activation, an ER transmembrane kinase, which will lead to inhibition of Eukaryotic Initiation Factor 2a (eIF2a) by phosphorylation hereby subsequently inducing the Activating Transcription Factor 4 (ATF4) transcriptional program. Deactivation of eIF2a will silence global mRNA translation to reduce the ER protein load [17]. ATF4 is generally regarded as a pro-survival regulator involved in drug resistance and its expression correlates with EMT [18,19].

Acriflavine (ACF) is a heteroaromatic dye with antibacterial and antiviral effects [20]. More recently, its potential as an anticancer agent emerged as acriflavine has topo-isomerase inhibitor activity [21]. It also blocks the HIF pathway, an important driver of cancer aggressiveness, by preventing the dimerization of the HIF-1 subunits, HIF-1α and HIF-1β [22]. Furthermore, there are also reports that show a close link between HIF signaling, EMT and cancer aggressiveness [7,8,23,24].

In this study, we show that acriflavine inhibits epithelial-to-mesenchymal transition induced by TGF-β1 or $CoCl_2$ (model of severe hypoxia) and in a model of acquired drug resistance. Furthermore, acriflavine blocks UPR via inhibition of the ATF4 transcriptional program and resensitizes drug resistant cells. These characteristics encourage further studies to repurpose ACF for systemic cancer therapy.

Methods

Cell Culture, EMT/UPR Induction and Acriflavine Treatment

Panc-1, human pancreatic cancer cells were obtained from ATCC (CRL-1469, Rockville, MD, USA). EMT was induced by transforming growth factor-β1 (TGF-β1, R&D systems, Minneapolis, USA) or cobalt chloride ($CoCl_2$) (Sigma-Aldrich, St. Louis, USA), the latter compound to mimic severe hypoxia. Experimentally, cells were first starved for 24 hours in medium with 1% fetal calf serum (FCS) to avoid interference from growth factors in the serum and then stimulated with TGF-β1 (5 ng/ml), $CoCl_2$ (200 μM) or vehicle for 48 hours. The development of the sorafenib-resistant cell line HepG2S1 (derived from human hepatoblastoma cell line HepG2 (ATCC, HB-8065) was done at the authors laboratory as described previously [12]. HepG2S1 have strong mesenchymal characteristics and are cultured continuously under drug selection with 6 μM of sorafenib (Bayer HealthCare, Leverkusen, Germany). Gene expression of the different lines was repeatedly determined over time by qRT-PCR and compared to transcriptome data of cells early after they were obtained from ATCC, this to monitor stability. The gemcitabine resistant human pancreatic cancer cell lines MiaPaCa-2 GR800 and their parental cell line MiaPaca-2 were a kind gift of Prof. T. Landowski [25] and were used to investigate the effect of ACF on drug sensitivity by XTT cell viability assay as described below.

To inhibit EMT in Panc-1 cells, acriflavine (2.5 μM unless otherwise specified) was added to the cell culture together with TGF-β1 or $CoCl_2$ for 48 h. As HepG2S1 cells show baseline EMT, acriflavine or vehicle was added to unstimulated cells for 24 h. To unravel the working mechanism of acriflavine, the compound was also added to unstimulated Panc-1 cells, for further details see Supplementary File.

Invasion Assay

Invasion studies were performed using BioCoat Matrigel Invasion Chambers (Corning, NY, USA). Cells (7.5×10^4) were added to the top inserts in FCS free medium with or without TGF-β1 (for Panc-1) and/or 2.5 μM of ACF (for both Panc-1 and HepG2S1). The bottom chambers were filled with medium containing 10% FCS, serving as a chemo attractant. After 48 h, the non-invaded cells were removed with a cotton swab and the assays were fixed and stained using DiffQuick (Medion Diagnostics AG, Düdingen, Switzerland). The number of invaded cells was determined under the microscope in five random fields per insert at 40× magnification.

Western Blot

Ten to fifty micrograms of protein per condition, obtained by cell lysis, were separated on a Mini-PROTEAN® TGX™ precast gel (BioRad, CA, USA). Proteins were transferred onto a nitrocellulose membrane and subsequently incubated with the appropriate primary and secondary antibodies (see Supplementary File). The immunoreactive bands were visualized using the enhanced chemiluminescent Western blot detection kit (BioRad). To verify equal protein loading, the blots were reprobed with β-actin antibody (Sigma-Aldrich). Images were visualized using the ChemiDoc™ MP Imaging System (BioRad).

RNA Isolation and qRT-PCR

RNA from three biological cell culture controls was isolated with the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. One microgram of cellular RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen/Life Technologies, USA). The PCR reaction was carried out in a mixture that contained appropriate sense- and anti-sense primers and a TaqMan MGB probe in Taq-Man Universal PCR Master Mixture (Applied Biosystems, Foster City, CA, USA) (see Supplementary File). Beta-2-microglobulin was used as housekeeping gene. qRT-PCR amplification and data analysis were performed using the Lightcycler 96 (Roche Applied Science, Penzberg, Germany). Each sample was assayed in duplicate. The ΔΔCq method was used to determine relative gene expression levels.

cDNA Microarray and RNA Sequencing and Data Processing

To explore changes at the transcriptome level under experimental conditions and to gain insights into the working mechanism of acriflavine, cell culture samples were analyzed using cDNA microarray (unstimulated Panc-1 cells and TGF-β1 experiment) or RNA sequencing (HepG2S1 and $CoCl_2$ experiment). Panc-1 cells were treated with 2.5 μM of ACF for 48 h (with or without concomitant stimulation). HepG2S1 cells were treated with 5 mM of ACF for 24 h. RNA was extracted as described above. The RNA

quality was assessed with the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). All samples were analyzed in triplicate (biological controls).

For microarray, the Affymetrix Human PrimeView Array was used as a platform. Gene expression data was analyzed with the Limma package from Bioconductor (http://www.bioconductor.org) [26]. Differentially expressed genes were assessed using a moderated *t* test.

For RNA sequencing, poly-A containing mRNA molecules were purified from the total RNA input using poly-T oligo-attached magnetic beads. Samples were sequenced on an Illumina NextSeq 500 half-flow cell. After preprocessing, reads were aligned with STAR 2.4.1d to the reference genome of *Homo sapiens* (GRCh37.73) [27] and counted with featureCounts 1.4.6 [28]. With the EdgeR 3.8.6 package of Bioconductor, generalized linear model (GLM) was fitted against the normalized counts [29]. The resulting p-values of the Limma and EdgeR packages were corrected for multiple testing with Benjamini–Hochberg to control false discovery rate [30]. A gene was considered differentially expressed if a 2log fold change > + 1 or <−1 and a corrected $P < .05$.

Microarray and RNA sequencing data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE82299.

The iRegulon tool identifies transcription factors regulating differentially expressed genes based on binding motifs and ChIP-seq data (tracks) [31]. We used this in silico analysis to predict the working mechanism of acriflavine. The genes differentially expressed in one direction upon stimulation and/or treatment were used as input. Only the transcription factors with the highest enrichment score were further evaluated. GSEA was performed of every experimental condition using the entire MSig database [32]. Only gene sets with enrichment *P* value <0.05 and FDR q-value <0.25 were considered significantly enriched (see Supplementary File).

Cell Viability Assay

Cell viability and proliferation was measured with the "Cell Proliferation Kit II (XTT)" (Roche). All experiments were performed at least three times with every condition in triplicate. Culture conditions were maintained in 96 well plates during 72 h (with refreshment of medium and compounds after 48 h). XTT solution was added to the wells at a concentration of 0.3 mg/mL and after 4 h of incubation with XTT assay, the metabolic activity as an indirect measure for cell number, was quantified spectrophotometrically at dual wavelength (490–655 nm). Results were expressed relative to control conditions.

Statistical Analysis

All statistics were performed using SPSS v23 (IBM). Statistical differences between groups were assessed with a Student's *t*-test or the Mann–Whitney Rank Sum Test when appropriate. For differences in gene expression assessed by qRT-PCR, ANOVA test with post hoc Tukey's procedure was used. A *P* value below .05 was considered statistical significant.

Results

Acriflavine Inhibits EMT

We investigated 3 mechanistically distinct models of EMT: (1) Panc-1 cells stimulated with TGF-β1, (2) Panc-1 cells exposed to chemically induced hypoxia ($CoCl_2$) and (3) HepG2 cells resistant to sorafenib (HepG2S1 cells). Light microscope confirmed loosening of cell–cell contact and the acquisition of a spindle-shaped morphology after stimulation of Panc-1 cells with TGF-β1 or $CoCl_2$ (Figure 1, *A* and *B*). On a functional level, stimulated Panc-1 cells acquired enhanced invasive capacities when assessed by Matrigel invasion assay (Figure 2*A*). Gene (qRT-PCR) and protein expression (Western blot) of EMT markers showed down-regulation of CDH1, a hallmark of EMT (Figure 3, *A* and *B*). These changes were accompanied by up-regulation of the EMT transcription factor SNAI1. Similar changes in EMT markers were seen in the $CoCl_2$ model (data not shown). Drug-resistant HepG2S1 cells underwent marked changes in morphology, invasive capacity and gene and protein expression compared to the parental HepG2 cells, compatible with pronounced EMT (Figures 1*C* and 2*B*) (as described previously (12)). Gene expression changes for the 3 models, as accessed by cDNA microarray or RNA sequencing, confirmed EMT characteristics (see GEO data GSE82299).

Next, we could demonstrate that acriflavine inhibits EMT. When cells were stimulated with TGF-β1 or $CoCl_2$ and simultaneously treated with ACF, they partially regained their epithelial characteristics, including closer cell–cell contact, this was most notably in the Panc-1/TGF-β1 model (Figure 1*A*). In contrast, no major change in morphology was noted in HepG2S1 cells treated with ACF alone (Figure 1*C*) (see discussion). ACF strongly inhibited the invasive potential of stimulated Panc-1 cells (Figure 2*A*) and HepG2S1 cells (Figure 2*B*). qRT-PCR and Western blot confirmed up-regulation of epithelial markers as well as down-regulation of mesenchymal markers upon ACF treatment (Figure 3, *A*–*C*). On the transcriptome level, acriflavine restored the expression of 191 of the 421 (45%) differentially expressed genes after EMT-induction by TGF-β1 stimulation and 214 of 611 (35%) genes by $CoCl_2$ stimulation (Figure 3*D*).

Acriflavine Restores Drug Sensitivity

We investigated whether acriflavine could enhance the sensitivity to cytotoxic drugs in the sorafenib-resistant HepG2S1 cells and gemcitabine resistant MiaPaCa-2 cells GR800. The latter cell line shows similar invasive characteristics and morphology as seen in HepG2S1 cells although without the typical marker expression profile of EMT [25]. In vitro, monotherapy with acriflavine at increased dose, was cytotoxic for all cell lines tested. However, the IC50 of ACF in the derived resistant cell lines; HepG2S1 and GR800 was higher compared to their parental cell lines; HepG2 and MiaPaCa-2, respectively (Figure 4). Treating resistant cells with an on itself non-toxic concentration of ACF leads to a significant enhancement of sorafenib sensitivity in HepG2S1 cells or that for gemcitabine in GR800 cells (Figure 4). These findings confirmed that ACF restores drug sensitivity in these models of mesenchymal differentiation upon acquired drug resistance.

Acriflavine Inhibits UPR Via ATF4

It is clear from several reports that acriflavine can have an effect on multiple cellular pathways [21,22]. To further unravel the mechanism of action of ACF, we assessed the gene expression changes upon ACF treatment in unstimulated Panc-1 cells (so without TGF-β1 or $CoCl_2$) and in HepG2S1 cells. Here, ACF led to remarkable inhibition of the unfolded protein response, demonstrated by gene set enrichment analysis (Figure 5*A*). The iRegulon tool pointed towards either ATF4, ATF3 or CEBPB as potential involved transcription factors of those genes down-regulated upon ACF treatment (Figure

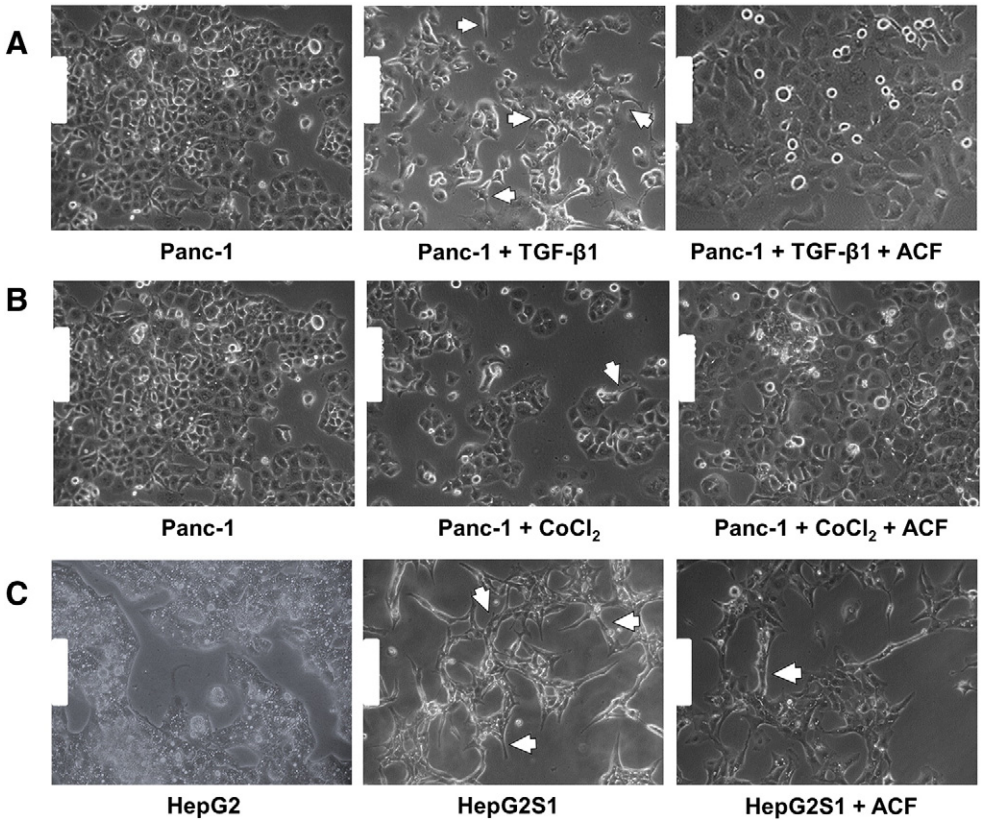


Figure 1. Morphological changes in the different EMT models before and after treatment with ACF. Cells were cultured in their appropriate medium. EMT was induced using TGF-β1 or $CoCl_2$ after 24 hour pretreatment with medium of 1% FCS (for Panc-1 cells) or after development of resistance against sorafenib (HepG2S1). Effect of ACF on morphology were observed under phase-contrast microscope on cells co-treated with a non-lethal dose of 2.5 μM ACF. The arrows indicate spindle shaped cells a morphological feature of EMT. Scale bars represent 200 μm. (For details see materials and methods).

5*B*) (see Supplementary File). As ATF4 is a master regulator of the UPR, we hypothesized that this transcription factor was indeed inhibited by ACF and not those regulators with similar binding motifs: ATF3 or CEBPB. Other arguments were derived from comparisons of our data with that of other published research. We found striking overlap between the genes down-regulated by knockdown of ATF4 in a lung cancer cell line [33] and genes down-regulated by ACF in the Panc-1 and HepG2S1 cell line (Figure 5*A*). Genes, down-regulated by ACF with an ATF4 binding motif in their regulatory control elements, were partially common and partially exclusive per cell type (Figure 5*C*). Gene sets containing members with promotor regions around the transcription start site of ATF3 or CEBPB were not significantly enriched or even significantly up-regulated after ACF treatment in Panc-1 or HepG2S1 cells (data not shown), suggesting that indeed ATF4 is most likely inhibited by ACF leading to UPR abrogation.

Acriflavine Inhibits UPR by Dephosphorylation of eIF2a

When cell lines are cultured in normoxia they have low baseline ER stress and limited unfolded protein response. We could confirm that chemically induced severe hypoxia indeed activated the UPR, which was inhibited by ACF (Figure 6*A*). ATF4 mRNA and protein levels were significantly up-regulated upon $CoCl_2$ stimulation and restored to baseline by ACF (Figure 6, *B* and *C*). ATF4 targets were significantly induced by $CoCl_2$ and inhibited by ACF (Figure 6*A*). Of note, expression of carbonic anhydrase 9 (CA9), a known target of ATF4 [34], was markedly induced by $CoCl_2$ and restored to baseline levels upon concomitant $CoCl_2$ stimulation and ACF treatment (Figure 6*B*). Low CA9 levels are associated with reduced tumor cell survival in acidic as well as hypoxic environment [35]. $CoCl_2$ also causes an amino acid deprivation response with, among others, up-regulation of tRNA synthetases. The expression of these ATF4 targets (a majority of the cytoplasmic forms and no mitochondrial tRNA synthetases) was strongly repressed by acriflavine (Figure 6*D*) (see also Supplementary File). More upstream of ATF4 in the PERK/eIF2a/ATF4 pathway, Western blot confirmed inhibition of eIF2a phosphorylation hereby tempering the unfolded protein response (Figure 6*C*). In line with previous findings [19], we found that EMT induction by TGF-β1 leads to activation of the PERK/eIF2a/ATF4 illustrated by phosphorylation of eIF2a and up-regulation of ATF4. However, the ATF4 transcriptional program is not activated to an extent comparable with $CoCl_2$ stimulation as evaluated by iRegulon and GSEA (data not shown). Nevertheless, ACF inhibits phosphorylation of eIF2a in this model thereby reducing ATF4 levels (Figure 6*C*).

Discussion

With this study, we provided evidence that acriflavine can interfere with EMT and UPR, two well-described pathways associated with

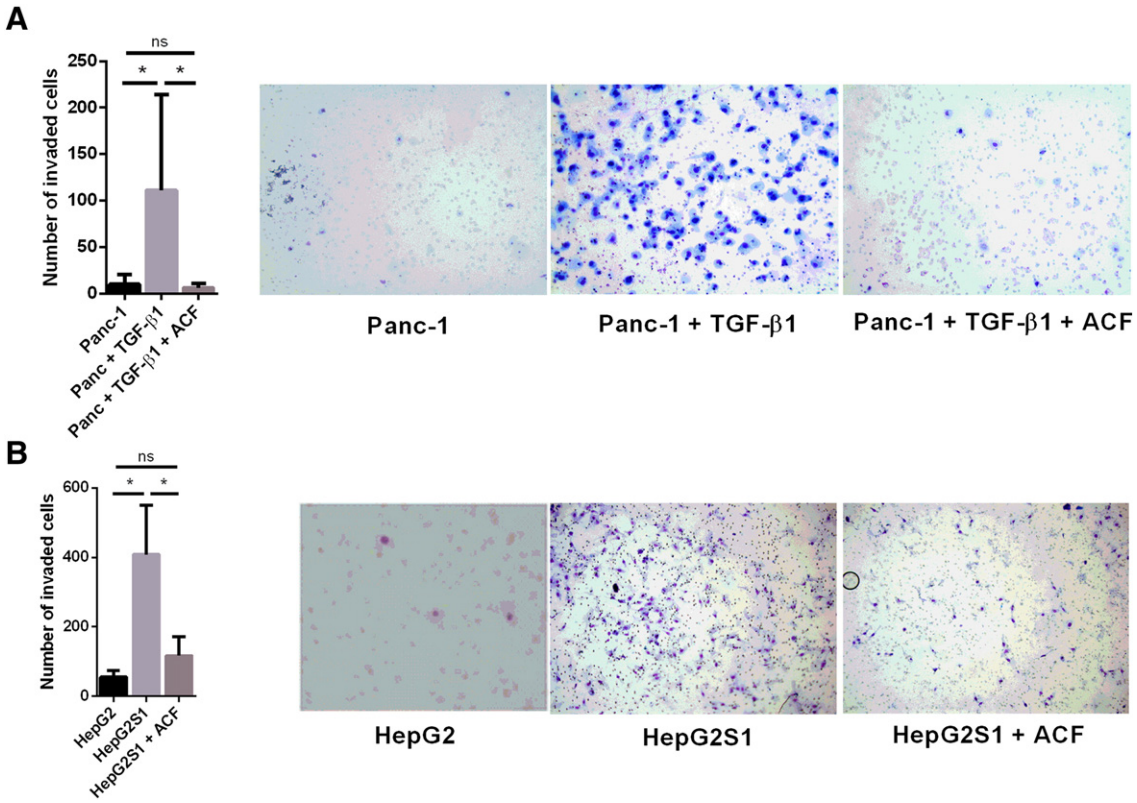
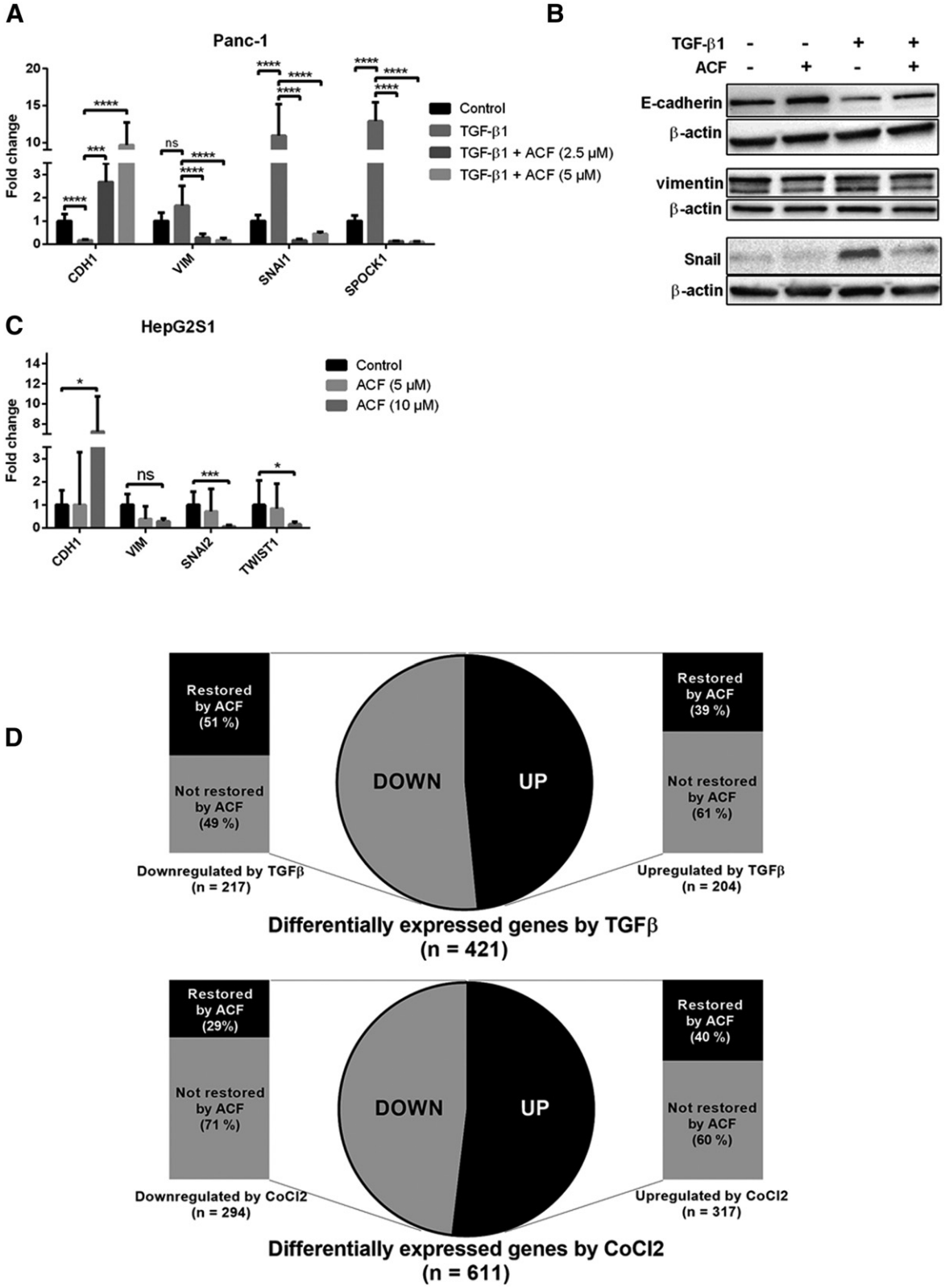


Figure 2. Invasive capacities of the EMT models before and after treatment with ACF. Invasion studies were performed as described in materials and method section. After 48 hours the cells that had invaded were fixed, stained and counted. The results are the mean of 3 biological repeats +/− SD. On the right side representative pictures of the microscopic view of the matrigel are given. Original magnification 10×, *: $P < .001$.

cancer aggressiveness. And, because drug resistance is also clearly associated with the mesenchymal state [36], blocking of the epithelial-to-mesenchymal transition is an attractive goal in ACF cancer therapy.

We could show that acriflavine, an old antibiotic recently identified as anticancer agent and HIF inhibitor [22], efficiently inhibits EMT in vitro. Expression of several important epithelial and mesenchymal markers that were altered by EMT induction can be restored using acriflavine (gene and protein level). At the morphological level, we showed that induced Panc-1 cells ($CoCl_2$ or TGF-β1: mesenchymal) following acriflavine treatment regain closer cell–cell contact as is seen in the non-stimulated Panc-1 cells (epithelial). In HepG2S1 cells, the phenotype is much more pronounced and "fixed" probably due to numerous genetic and epigenetic alterations present in these cells, which was already shown in similar models of mesenchymal differentiation upon acquired drug resistance [37]. Nevertheless, the invasive capacities of stimulated Panc-1 cells and of HepG2S1 could be abrogated by a noncytotoxic concentration of acriflavine. Much more than the expression of a single epithelial or mesenchymal marker, this effect on the cellular behavior is crucial to demonstrate EMT inhibitory activity.

Another remarkable effect of acriflavine is the inhibition of the unfolded protein response. The role of the UPR is to limit cellular damage during stress, explaining why the UPR has been linked to cancer drug resistance [18]. However, this mechanism equally drives the cell to apoptosis when the stress is too severe [38]. Given this dual role, the question rises whether either inhibition or stimulation of the UPR for cancer therapy is most desirable. With this study, we showed that acriflavine causes dephosphorylation of eIF2a, hereby inhibiting the ATF4 transcriptional program. ATF4 is one of the central transcription factors in the UPR induced by severe hypoxia or anoxia, independent of HIF signaling [39]. ATF4 is generally regarded as a pro-survival factor associated with resistance to anti-cancer drugs, which makes it an attractive target [18]. Several approaches to target ATF4 have been suggested, including the inhibition of upstream factors PERK and eIF2a [40]. To the best of our knowledge, no drugs have yet demonstrated to clearly abrogate the ATF4 transcriptional program. Moreover, several genes regulated by ATF4 and affected by ACF, such as carbonic anhydrase 9, are attractive targets for cancer therapy [41]. Acriflavine can thus be considered as a broad hypoxia pathway inhibitor in in vitro cancer models. Earlier, the drug was identified as a HIF inhibitor by preventing dimerization of the two HIF-1 subunits [22]. We now show that ACF also blocks the alternative hypoxia pathway via ER stress causing abrogation of hypoxic cell survival [42]. To be noted, ACF suppresses predominantly the expression of cytoplasmic aminoacyl t-RNA synthetases and not the mitochondrial forms (see Supplementary Table 4) suggesting that the mitochondria and energy supply of the cell are not affected. Furthermore, in two models, hepatoma cells resistant to sorafenib and pancreatic cancer cells resistant to gemcitabine, we



could demonstrate a shift of drug sensitivity towards the parental cell line. Together, these action mechanisms of ACF result in diminished invasion and resensitization of cancer cells in vitro.

Being an in vitro study it has some limitations before it can be translated to the clinic. We investigated several cell models in which the results on EMT or ACF are not always identical. We selected the models to be complementary, we saw that EMT induction by stimulation (TGF-β1 or $CoCl_2$) is functional not very different from the drug resistance models although certain molecular features are not exactly the same. The same variation is to be expected when in vivo different microenvironmental factors induce a cancer cells to EMT. We then looked for features that could be repeated in the different

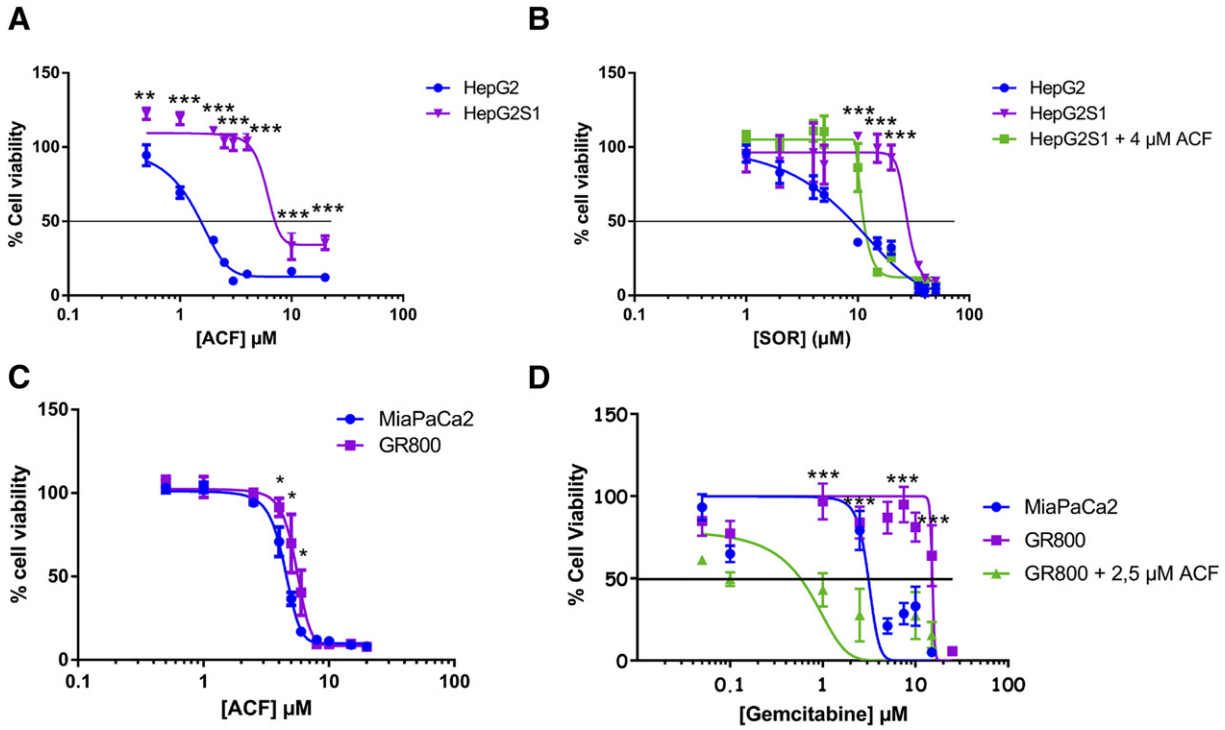


Figure 4. ACF partially restores drug sensitivity in models of acquired resistance. Dose dependent cell death was investigated using XTT assay as described in materials and methods. A) Sensitivity of HepG2 and derived sorafenib-resistant HepG2S1 cells to ACF. B) ACF at a non-lethal dose restores sorafenib sensitivity in HepG2S1 cells. C) Sensitivity of MiaPaCa-2 and derived gemcitabine resistant cell line GR800 to ACF. D) Similarly, ACF restores gemcitabine sensitivity in GR800 cells.

models of aggressive cancer. Our conclusions are therefore based on the linkage of transcriptome data with alterations in cellular programs EMT and UPR. Although these findings are very suggestive for a causal relationship, additional mechanistic studies are needed to definitely confirm our hypotheses. However, recent studies have shown that PERK/eIF2a activation is necessary for EMT cells to invade and metastasize providing a link between the two concepts [19]. Moreover, ATF4 knock-down impedes hypoxia induced EMT in gastric cancer cell lines [16].

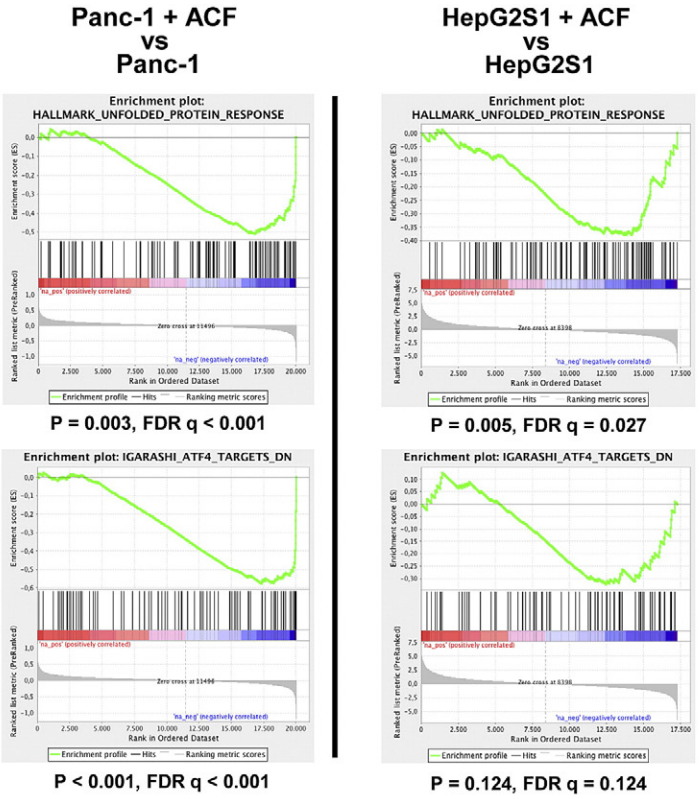
From these and other studies, it is clear that acriflavine is an interesting compound with pleiotropic anticancer effects [21,22,43]. Its past systemic use in the clinical setting as an antibiotic without any major toxicity reported encourages further development of the drug for cancer treatment [44]. We hypothesize that ACF pushes the cancer cell to an epithelial state, blocking the development of drug resistance and prolonging the time frame over which a drug can have its effect on the tumor and so increasing its effectiveness. However, at present no preparation for clinical use is available and the interest of the industry in off-patent drugs is limited [45]. We believe studies like these should prompt non-profit institutions to take initiatives that allow repurposing acriflavine for animal and clinical testing in oncology.

Acknowledgements

We acknowledge our use of the gene set enrichment analysis, GSEA software, and Molecular Signature Database (MSigDB) (Subramanian, Tamayo, et al. (2005), PNAS 102, 15545-15550, http://www.broad.mit.edu/gsea/). We thank prof. Landowski for providing us with the gemcitabine resistant MiaPaCa-2 cell lines and prof. Stein Aerts and his colleagues for the discussion on the iRegulon results. CV holds a mandate as Senior Clinical Investigator of the Research Foundation - Flanders (Belgium) (FWO). This study was partly

Figure 3. Gene and protein expression analysis in models of EMT with and without treatment with ACF. (A) Gene expression was determined in the Panc-1/TGF-β1 model. After 24 hours of growth in medium with 1% FCS followed by 48 hours stimulation with or without ACF the cells were harvested and qRT-PCR performed. (B) Protein expression by Western blot of marker genes for EMT: E-cadherin, Vimentin and Snai1. Representative blots are shown here. (C) Gene expression by qRT-PCR of sorafenib-resistant HepG2S1 cells treated with different concentrations of ACF for 24 hours. (D) ACF restores the expression of a significant fraction of genes in the EMT models. We analyzed the genes that were differentially expressed by TGF-β1 or $CoCl_2$ treatment in Panc-1 cells for the effect of co-treatment with ACF using transcriptome data. Upon TGF-β1 stimulation 421 genes were significantly changed in expression (2log change > +1 or <−1 and p_{corr}< 0.05). Of these, ACF restored 45% of differentially expressed genes (top). In the $CoCl_2$ stimulated cells we found 611 genes to be differentially expressed, here ACF restored 35% (bottom).All experiments were performed in 3 biological repeats. qRT-PCR results are given as mean fold change +/− SD. (*: $P < .05$, **: $P < .01$, ***: $P < .001$ and ****: $P < .0001$).

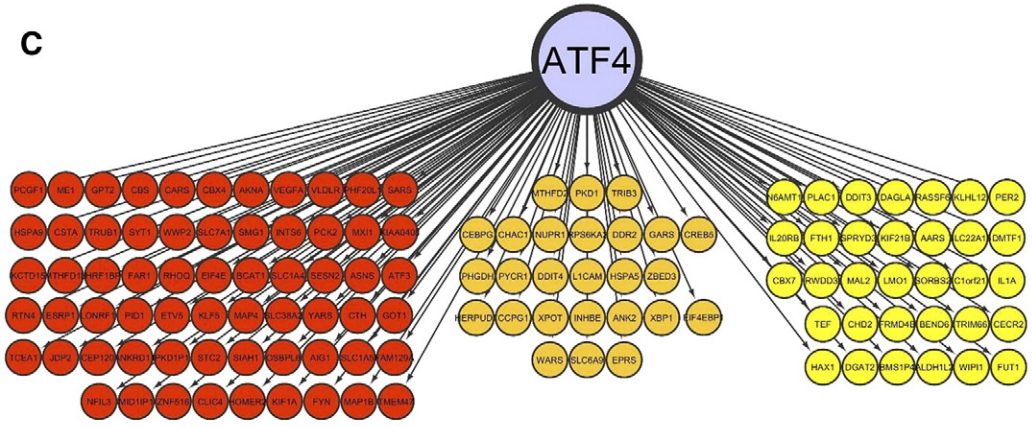
A



B

Regulators of genes DOWN-regulated by ACF in Panc-1 cells			Regulators of genes DOWN-regulated by ACF in HepG2S1 cells		
By motifs					
AUC	NES	Transcription factor	AUC	NES	Transcription factor
0.111	12.643	ATF4,ATF3,CEBPB	0.029	5.036	ATF4,ATF3,CEBPB

C



Down-regulated by ACF in Panc-1 cells

Down-regulated by ACF in HepG2S1 cells

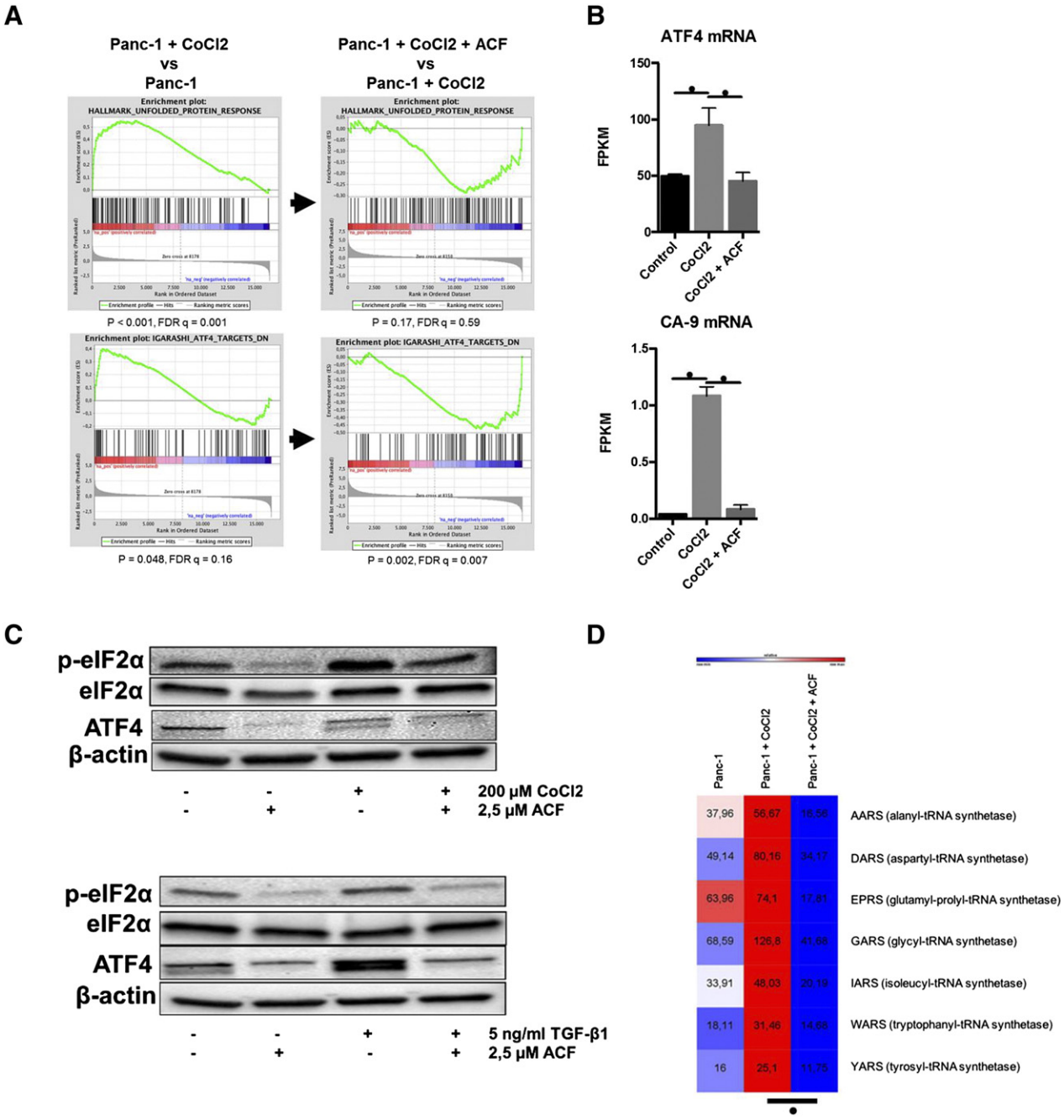


Figure 6. ACF inhibits UPR in Panc-1 model induced by $CoCl_2$. (A) GSEA analysis of the transcriptome data showed that ACF inhibits UPR induced by $CoCl_2$. $CoCl_2$ stimulation leads to ATF4 activation which is abrogated by concomitant ACF treatment. (B) ATF4 mRNA levels and its target gene CA-9 are up-regulated by $CoCl_2$ and restored to baseline by ACF. (C) On Western blot, phosphorylation of eIF2a caused by either $CoCl_2$ or TGF-β1 is inhibited by ACF. D) tRNA synthetases are induced by $CoCl_2$ treatment and down-regulated by ACF. Numbers represent FPKM (Fragments Per Kilobase Million) values.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.11.008.

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Figure 5. ACF inhibits the unfolded protein response and ATF4 transcriptional program. Transcriptome data were analyzed using two software programs iRegulon and GSEA. (A) GSEA analysis ranked unfolded protein response (upper row) and ATF4 targets (lower row) in Panc-1 cells and HepG2S1 models as highly significantly enriched upon treatment with ACF. (B) iRegulon identifies ATF4, ATF3 or CEBPB as regulating transcription factor in the working mechanism of ACF in Panc-1 and HepG2S1 cells. (C) ATF4 targets significantly down-regulated by ACF in Panc-1 cells, HepG2S1 cells or both. (AUC, area under the curve; NES, normalized enrichment score).

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